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# Connection between the membrane electron transport system and Hyn hydrogenase in the purple sulfur bacterium, *Thiocapsa roseopersicina* BBS



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## ABSTRACT

*Thiocapsa roseopersicina* BBS has four active [NiFe] hydrogenases, providing an excellent opportunity to examine their metabolic linkages to the cellular redox processes. Hyn is a periplasmic membrane-associated hydrogenase harboring two additional electron transfer subunits: Isp1 is a transmembrane protein, while Isp2 is located on the cytoplasmic side of the membrane. In this work, the connection of HynSL to various electron transport pathways is studied. During photoautotrophic growth, electrons, generated from the oxidation of thiosulfate and sulfur, are donated to the photosynthetic electron transport chain via cytochromes. Electrons formed from thiosulfate and sulfur oxidation might also be also used for Hyn-dependent hydrogen evolution which was shown to be light and proton motive force driven. Hyn-linked hydrogen uptake can be promoted by both sulfur and nitrate. The electron flow from/to HynSL requires the presence of Isp2 in both directions. Hydrogenase-linked sulfur reduction could be inhibited by a  $Q_B$  site competitive inhibitor, terbutryne, suggesting a redox coupling between the Hyn hydrogenase and the photosynthetic electron transport chain. Based on these findings, redox linkages of Hyn hydrogenase are modeled.

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## 1. Introduction

In microbial cells, hydrogenases and nitrogenases can catalyze  $H_2$  evolution. Hydrogenases are dedicated enzymes to reversible oxidation of molecular hydrogen to protons and electrons. Hydrogenases are classified according to the metal content of their active center: [Fe], [FeFe] and [NiFe] hydrogenases [1]. The functional core of [NiFe] hydrogenases is a heterodimer, the [NiFe] active center is located in the large subunit, while the small subunit contains iron–sulfur clusters playing a role in electron transport [1]. Hydrogenases usually contain additional subunits which link the core subunits to the redox processes of the cell [2]. Hydrogenases might have various physiological roles, such as donating electrons from molecular hydrogen to  $NAD^+$  or another electron acceptor, such as fumarate, nitrate, metals and sulfate. Electron transfer subunits couple the core enzyme to the cellular redox processes. The types of these additional subunits might be informative with regard to the physiological role of the enzyme [1]. Hydrogenases can evolve hydrogen in vivo in order to facilitate the cofactor regeneration and decrease the overreduction of the membrane redox system [3,4].

*Thiocapsa roseopersicina* BBS is a purple sulfur photosynthetic bacterium belonging to the *Chromatiaceae* family. During anoxygenic growth,

it uses thiosulfate, sulfide, elemental sulfur and polysulfide as electron donors for carbon dioxide fixation [5]. The draft genome sequence of *T. roseopersicina* was determined and annotated. Based on the genomic data, it is assumed that assimilation of thiosulfate is carried out by the periplasmic thiosulfate oxidizing multienzyme complex (Sox) according to the models based on *Paracoccus pantotrophus* and *Allochrochromatium vinosum* [6,7]. In the Sox cycle of *A. vinosum*, two electrons are donated to the cytochrome c of the photosynthetic electron transport system and sulfur is deposited in sulfur globules [8].

The oxidation of endogenous or exogenous sulfur in *T. roseopersicina* seems to be analogous to the processes taking place in *A. vinosum* [KJ179956] [36]. After transportation of stored sulfur to the cytoplasmic DsrC [9,10], the bound sulfur is oxidized to sulfite via the DsrAB dimer. According to the model, four electrons enter into the photosynthetic electron transport chain via the DsrMKJOP complex [11–13].

In the model, further oxidation of sulfite to sulfate might be catalyzed by the adenosine-5-phosphosulfate reductase enzyme (APS) [14] and ATP sulfurylase [15]. Sulfite is oxidized by the SoeABC complex [KJ194602], which transfers electrons to the quinone pool [16].

There are several reactions linked to the reduction of quinone pool, such as sulfide oxidation via SQRs, succinate oxidation to fumarate via succinate:quinone oxidoreductase and sulfite oxidation to sulfate via SoeABC. SQRs and succinate:quinone oxidoreductases are widespread among microbes and their genes were identified in the *T. roseopersicina* genome, as well. The electrons stored in the quinone pool can be used for  $NAD^+$ , nitrate and various sulfur compounds reduction [17,18,19,20].

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*T. roseopersicina* contains four active [NiFe] hydrogenases. Two are cytoplasmic NAD<sup>+</sup>-reducing enzymes, Hox1 [21] and Hox2 [22], the other two enzymes (HupSL, HynSL) are bound to the membrane [2]. Hox2 could be detected only in the presence of glucose [22]. Hox1 can evolve H<sub>2</sub> in vivo from various sources, e.g. from sulfur compounds under illumination and from catabolism of organic substrates in the dark. Therefore, it has connections to several metabolic pathways, as was suggested in previous studies [3,4].

Among the membrane bound enzymes, HupSL is an uptake hydrogenase, which oxidizes H<sub>2</sub> yielding protons in the periplasm and electrons. The proton gradient formed is used for ATP synthesis while the electrons are transferred to the central quinone pool via HupC protein. In contrast to *Wolinella succinogenes*, a Hup hydrogenase-linked sulfur reduction was not observed in *T. roseopersicina* [23]. The Hyn enzyme is a bidirectional membrane-associated periplasmic hydrogenase which has remarkable activity at high temperature [24]. In the *hyn* operon (ID:AF002817), there are two ORFs between the *hynS* and *hynL* genes, named *isp1* and *isp2*. These ORFs encode two proteins, Isp1 and Isp2 [2,25]. Isp1 is predicted to be a transmembrane protein with a di-heme binding site, while Isp2 seems to be a hydrophilic component of the membrane-bound complex which resembles the heterodisulfide reductase D protein of methanogen strains [26]. Isp2 has a remarkable similarity to the DsrK subunit of the sulfur-oxidizing system of purple sulfur bacteria [36]. An *isp2*-like gene is also part of the membrane-bound hydrogenase gene cluster in *Acidianus ambivalens*. In this organism, this hydrogenase is a component of a supercomplex together with a membrane-bound periplasmic sulfur reductase [17]. It should be noted that quinone binding sites could not be identified in either Isp1 or Isp2 using computational approaches [2,25].

In *Aquifex aeolicus*, an in vitro study pointed out that the hydrogen-dependent sulfide production, catalyzed by a membrane-bound hydrogenase/sulfur reductase complex, was linked to quinones as electron carriers [28]. Hydrogen-linked sulfur reduction was found in several other microbes, such as in *W. succinogenes* where a Hup-type hydrogenase and hydrogen-driven sulfur reduction was described both in vivo and in vitro [23]. The two systems have remarkable similarities; a) in both cases, hydrogen is the electron donor and b) a periplasmic sulfur reductase belonging to molybdoprotein family is responsible for hydrogen sulfide production and c) quinones are used as electron carriers.

Due to its special electron transfer subunits [2,25], quite limited information is available about the physiological role and redox partners of the Hyn complex in *T. roseopersicina*. The bidirectional character of this enzyme and its relation to H<sub>2</sub>S production were phenomenologically described in previous studies [2,3,27], but the molecular background of the redox processes remained unknown.

In this work, the redox routes coupled with HynSL hydrogenase were studied with a special emphasis on the metabolic context of HynSL, sulfur metabolism and photosynthesis. Moreover, the role of the Isp2 subunit in these redox processes is also discussed.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and primers

Bacterial strains, plasmids used in this study are listed in Table 1A and B. In Table 1C, the names and the sequences of the primers employed are displayed.

### 2.2. Cultivation conditions

*T. roseopersicina* strains were grown photoautotrophically in modified Pfennig's medium under anaerobic conditions with continuous illumination (50 µE) at 28 °C. The nitrogenase was repressed with NH<sub>4</sub>Cl; Na<sub>2</sub>S was usually omitted from the basic medium. Otherwise, the actual compositions of media are indicated in the description of

**Table 1**

Strains (A), constructs (B) and primers (C) used in this study.

| Name                        | Genotype or phenotype   | Source or reference     |
|-----------------------------|---|-------------------------|
| <b>A. Strains:</b>          |   |                         |
| GB2131                      | <i>hupSL::Gm<sup>r</sup>, hoxH::Em<sup>r</sup>, erm(Em<sup>r</sup>)</i><br>oriented as <i>hox</i>   | [21]                    |
| GB1121                      | <i>hupSL::Gm<sup>r</sup>, HynS::Sm<sup>r</sup></i>  | [21]                    |
| GB112131                    | <i>hynSL::Sm<sup>r</sup>, hupSL::Gm<sup>r</sup>, hoxH::Em<sup>r</sup>, erm(Em<sup>r</sup>)</i> oriented as <i>hox</i>   | [21]                    |
| THOE5M (GB112131 + pTHOE5M) | <i>hynSL::Sm<sup>r</sup>, hupSL::Gm<sup>r</sup>, hoxH::Em<sup>r</sup>, erm(Em<sup>r</sup>)</i> oriented as <i>hox</i> + pTHOE5M plasmid                                 | [27]                    |
| Isp2M                       | GB2131Δ <i>isp2</i>   | This work               |
| Isp2MpDSKIsp2               | <i>Isp2M</i> + pDSKIsp2 plasmid   | This work               |
| XL1-Blue MRF <sub>-</sub>   | Δ( <i>mcrA</i> )183 Δ( <i>mcrCB-hsdSMR-mrr</i> ) 173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI<sup>q</sup>ZΔM15 Tn10</i> (Tet <sup>r</sup> )]c | Stratagene              |
| S17-1(Δ <i>pir</i> )        | 294 ( <i>recA pro res mod</i> ) Tp <sup>r</sup> Sm <sup>r</sup> (pRP4-2-Tc::Mu-Km::Tn7) Δ <i>pir</i>  | [31]                    |
| <b>B. Plasmids.</b>         |   |                         |
| pDSK509                     | bhr cloning vector Km <sup>r</sup>  | [32]                    |
| pTHOE5M                     | pDSK509 with <i>hynS-isp1-isp2-hynL</i>   | [27]                    |
| pK18Isp2M                   | EcoRI-XbaI fragment of pBtlsp2M + pK18MobSacB   | This work               |
| pK18MobSacB                 | Km <sup>r</sup> <i>sacB</i> RP4 <i>oriT</i> ColE1 <i>ori</i>  | [33]                    |
| pDSKIsp2                    | pDSK5crtKm + NdeI-BamHI fragment of pBIS2   | This work               |
| pBluescript SK(+) + pBIS2   | Amp <sup>r</sup> , cloning vector, ColE1 pBluescript SK(+) + <i>isp2o1-isp2o2</i> PCR product in SmaI site  | Stratagene<br>This work |
| pDSK5crtKm                  | Crt promoter in pDSK509   | This work               |
| pBtlsp2M                    | pBluescript SK(+) + <i>otsh5r-otsh2o4</i> PCR product to BamHI site + <i>isp2o9-trhynl2</i> in EcoRV site   | This work               |
| <b>C. Primers:</b>          |   |                         |
| Name                        | Sequence 5' 3'  |                         |
| otsh5r                      | GGCTGCTCCGAGCCGAG   |                         |
| otsh2o4                     | GCACGCAGCTCGAAAAGAAG  |                         |
| isp2o9                      | GAGATCCCGGTGGTCGCTCTC   |                         |
| trhynl2                     | ACGTATTCTCGGATCTGACC  |                         |
| isp2o1                      | ATGAGCGAGCTGACACTTG   |                         |
| isp2o2                      | GGTCTCGGCGATCAT   |                         |

the given experiment. In the case S<sup>0</sup>-containing media, colloidal elemental sulfur (Riedel de Haen) was used.

The acetate-supplemented plates (2 g/L) were solidified with 7 g/L of phytagel (Sigma, St Louis, MO, USA). The plates were incubated in anaerobic jars by means of the Anaero Cult (Merck, Darmstadt, Germany) system for 2 weeks. The *Escherichia coli* strains were maintained on LB-agar plates. Antibiotics were used in the following concentrations for *T. roseopersicina* (mg/L): erythromycin (25), kanamycin (25), streptomycin (5) and gentamicin (5); for *E. coli*: ampicillin (100) and kanamycin (25),

### 2.3. Construction of Isp2M strain

The downstream homologous region of *isp2* was amplified using the *otsh5r-otsh2o4* primers and cloned into the BamHI site of pBluescript SK(+) (Stratagene) after blunting (pBtl2D). The upstream homologous region obtained by PCR applying the *isp2o9* and *trhynl2* primers was inserted into the EcoRV site of pBtl2D resulting in pBtlsp2M. The EcoRI-XbaI fragment of pBtlsp2M was cloned into pK18mobsacB yielding pK18Isp2M. This was introduced into S17-1 (Δ*pir*) and conjugated into GB2131 (Isp2M strain). Conjugation protocol was carried out as described earlier [22]. The genotype was confirmed by PCR reaction, Southern blotting and hybridization.

#### 2.4. Homologous complementation of *isp2* deletion

The product obtained by PCR using the *isp2o1*–*isp2o2* primers was ligated into the *Sma*I site of pBluescript SK(+) (pBIS2). The *Nde*I–*Bam*HI fragment of pBIS2 was inserted into corresponding sites of pDSK509CrtKm5 yielding pDSK1sp2. This was introduced into *Isp2M* strain to restore the phenotype of GB2131.

#### 2.5. Hydrogenase activity measurements

Hydrogenase activities of the cells were measured *in vivo*, as described earlier [2,21,22]. In all experiments, the GB112131 strain ( $\Delta hoxH$ ,  $\Delta hupSL$ ,  $\Delta hynS$ –*isp1*–*isp2*–*hynL*) [27] was used as negative control.

#### 2.6. Cultivation conditions for *in vivo* hydrogen evolution activity measurements without media change

First, the thiosulfate and carbonate contents of the media were optimized for obtaining clear demonstrative picture about the processes. Cultures were grown in 125 mL septa covered serum-vials in modified Pfennig's media containing 5 mM  $\text{NiCl}_2$  in the presence of various amounts of sodium thiosulfate and 23 mM sodium hydrogen carbonate; the gas phase was flushed with  $\text{N}_2$  after inoculation and on the 3rd day. The  $\text{H}_2$  produced was measured with gas chromatography on the 6th day [29].

For measuring  $\text{H}_2$  evolution in the presence of exogenous elemental sulfur as sole electron source, 3 mM sodium hydrogen carbonate containing media was used (100 ml culture volume in 125 ml serum-vial). The headspace was flushed with nitrogen after inoculation and on day 4. The  $\text{H}_2$  content of the gas phase was determined on the 6th day as above.

#### 2.7. Cultivation conditions for measurements performed after media change

Cells were propagated in the presence of 60 mM (for intensive sulfur globule accumulation) and 9 mM (to prevent sulfur globule accumulation) sodium thiosulfate and 24 mM sodium hydrogen carbonate for 4 days under continuous illumination. Cells were pelleted with centrifugation 9000 rpm for 10 min, 4 °C, washed with thiosulfate- and carbonate-free media (pH was adjusted to 7.0). After the second pelleting, cells were resuspended in sodium thiosulfate and sodium hydrogen carbonate-free media. Hydrogen and hydrogen sulfide were measured daily.

#### 2.8. Photosynthetic electron transport inhibitor and uncoupler studies

Inhibition experiments were carried out in serum vials containing 12.5 ml 8-fold diluted cell cultures. The GB2131 cells were grown in the presence of 60 mM thiosulfate (intensive sulfur globule accumulation) for 3 days, cells were harvested, then resuspended in thiosulfate- and carbonate-free media and supplemented with terbutryne (200  $\mu\text{M}$  final concentration) or its solvent (50  $\mu\text{L}$  DMSO). Then, the samples were flushed with hydrogen and kept in darkness for 12 h to allow the formation of  $\text{H}_2\text{S}$ .

CCCP (Carbonyl cyanide *m*-chlorophenyl hydrazine) was applied in the concentration range of 2.5–20  $\mu\text{M}$ . In these experiments, the THOE5M strain was used and the harvested cells were resuspended in either carbonate- and thiosulfate-free or carbonate-free but thiosulfate-containing media. The samples were flushed with  $\text{N}_2$  and the  $\text{H}_2$  evolved was measured after 24 h. Prolonged measurement was not possible due to the instability of CCCP. Since significantly fewer cells were used for a shorter period, the absolute values of the  $\text{H}_2$  content cannot be compared to other experiments.

For inhibition of cytochrome bc1 complex, myxothiazol/antimycin A cocktails were used in the following concentrations: 0.6  $\mu\text{M}$  Myx/2.5  $\mu\text{M}$

Ant, 1.25  $\mu\text{M}$  Myx/5.0  $\mu\text{M}$  Ant, 2.5  $\mu\text{M}$  Myx/10.0  $\mu\text{M}$  Ant and 5.0  $\mu\text{M}$  Myx/20  $\mu\text{M}$  Ant. The cells were treated similarly to the CCCP experiments, but the  $\text{H}_2$  formation was monitored for three days.

#### 2.9. Determination of hydrogen sulfide

The presence of  $\text{H}_2\text{S}$  was determined by gas chromatography on the 6th day as follows: samples were injected into Shimadzu GC-2010 gas chromatograph equipped with a TCD, and J&W Scientific Plot-Q column (30 m  $\times$  0.53 mm  $\times$  40  $\mu\text{m}$ , oven temperature 120 °C, inlet pressure 81 kPa. Split ratio 0.5:1, Inlet temperature: 200 °C, TCD temperature: 200 °C).

#### 2.10. Western-hybridization

Strains grown on standard nickel-containing Pfennig's medium with sodium thiosulfate (9–48 mM) were used. The protein content of the cell extracts, prepared by sonication [27] and TCA precipitation, was quantified by Micro-Lowry method [30]. NuPage BisTris 10% gel was used to separate proteins (25  $\mu\text{g}$  in each lane). Running, transfer blocking and developing happened as described elsewhere [27]. Bands were detected and analyzed by Bio-Rad VersaDoc 4000 gel documenting system.

### 3. Results and discussion

#### 3.1. Electron donors of Hyn hydrogenase

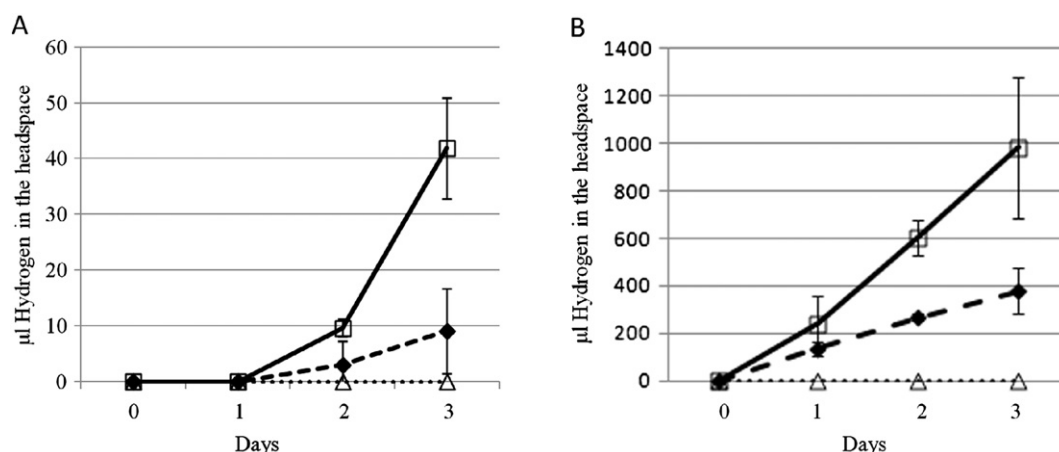
Hyn hydrogenase is a bidirectional enzyme associated with special additional subunits, *Isp1* and *Isp2* [2,27]. The physiological function of this heterotetrameric complex is still to be disclosed. Sodium thiosulfate is the primary electron source of *T. roseopersicina* BBS and its utilization is accomplished by elemental sulfur formation. Both thiosulfate and  $\text{S}^0$  are able to donate electrons to the photosynthetic electron transport chain, consequently they are electron donors of carbon dioxide fixation and central redox processes required for growth. They can also provide electrons for Hox1-mediated hydrogen evolution [21].

Two strains were constructed and used for testing the hydrogen evolution capacity of Hyn. One is GB2131 ( $\Delta hup$ ,  $\Delta hox$ ) while the other is the THOE5M strain containing a plasmid borne Hyn in the GB112131 ( $\Delta hyn$ ,  $\Delta hup$ ,  $\Delta hox$ ) strain (see Table 1) [27]. The GB112131 cell line was used as negative control. Under the conditions used, the activity of Hox2 could not be detected [22]. After testing various thiosulfate concentrations, we found that high amount of sodium thiosulfate could provide enough reducing equivalents for the Hyn hydrogenase-catalyzed  $\text{H}_2$  evolution in the GB2131 strain. For example, the accumulative hydrogen evolution of GB2131 grown photoautotrophically in the presence of 36 mM sodium thiosulfate was 50  $\mu\text{L}$  between day 3 and 6. In contrast, no hydrogen evolution could be detected in samples grown in medium supplemented with 12 mM thiosulfate or in the case of the control strain, GB112131 at any thiosulfate concentration.

According to the model described for the thiosulfate assimilation of *A. vinosum*, two electrons are gained, while sulfate and elemental sulfur are formed [6,7]. Oxidation of sulfur to sulfite releases four electrons and another two electrons might be obtained during the oxidation of sulfite to sulfate [34]. The following question was addressed: which step(s) can donate electrons to HynSL?

THOE5M, GB2131 and GB112131 strains were grown in a medium promoting intensive sulfur globule accumulation (containing 60 mM thiosulfate and 23 mM hydrogen carbonate). The cells were centrifuged, resuspended in a carbonate- and thiosulfate-free media and kept under illumination. Hyn hydrogenase could evolve  $\text{H}_2$  from the endogenous sulfur as sole electron donor but externally added thiosulfate dramatically increased the  $\text{H}_2$  evolution (Fig. 1). This suggests that both thiosulfate and sulfur oxidation could provide electrons to Hyn simultaneously.

Significantly higher amount of  $\text{H}_2$  could be produced by the recombinant Hyn enzyme under these conditions (Fig. 1). The expression level



**Fig. 1.** Light-driven  $H_2$  evolution of Hyn-containing strains after media change. A) GB2131; B) THOE5M. The media contained: endogenous elemental sulfur (dashed line, ♦ symbol); endogenous elemental sulfur and 4 mM sodium thiosulfate (continuous line, □ symbol); 4 mM sodium sulfite as sole electron donor in the absence of stored elemental sulfur (dotted line, Δ symbol) under illumination.

of HynSL in the GB2131 (*hyn* genes in the genome) and the THOE5M (*hyn* genes in a multicopy vector) strains were compared by Western analysis. Much more hydrogenase protein could be observed in the THOE5M strain than in the GB2131 (data not shown). This might explain the elevated  $H_2$  production of the THOE5M strain. These results also indicated that the amount of Hyn hydrogenase might be a limiting factor in  $H_2$  evolution at high electron flux, at high substrate concentrations. It is notable, that thiosulfate had no effect on the expression level of Hyn in either cell line (data not shown).

From these experiments, it could be concluded that oxidation of both sulfur and thiosulfate can provide electrons for Hyn hydrogenase. Since sulfur oxidation produces sulfite, as intermediate, the next question was whether sulfite oxidation could drive the Hyn-coupled  $H_2$  evolution. Cells were cultivated in the presence of 9 mM thiosulfate. Under these conditions, sulfur globules were not formed. Then, the media was replaced with a thiosulfate- and carbonate-free solution which was supplemented with sodium sulfite as sole electron donor and the  $H_2$  content of the headspace was monitored. No  $H_2$  could be detected, consequently sulfite oxidation cannot deliver electrons for Hyn under the conditions used (Fig. 1A and B dotted lines).

Several other potential electron donors (succinate and glucose), which could be used for photobiohydrogen production by *T. roseopersicina* in nitrogen-fixing conditions [35], were tested under phototrophic conditions. Neither glucose nor succinate, nor any other organic acid could promote  $H_2$  evolution by Hyn (data not shown).

### 3.2. Sulfide and hydrogen formation is a competitive process

Hydrogen evolution is not the only way to eliminate the excess reducing power. During sulfur oxidation in carbonate-depleted cultures, either  $H_2$  or  $H_2S$  can be evolved.  $H_2S$  formation was monitored in the GB2131 and THOE5M cultures cultivated in the presence of 36 mM thiosulfate and incubated in thiosulfate- and carbonate-free media as described in Section 2.6.

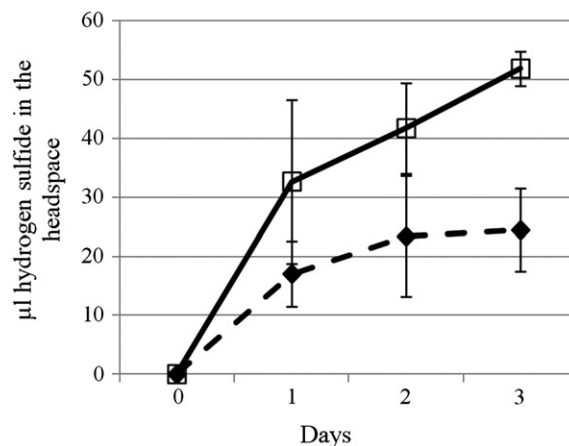
Both GB2131 and THOE5M strains produced  $H_2S$ , but  $H_2S$  production in the THOE5M strain was apparently lower than that in the GB2131 strain [Fig. 2]. Thus, THOE5M produces more hydrogen and less hydrogen sulfide as compared to the data obtained for GB2131. It is difficult to establish the exact stoichiometry of the increase in  $H_2$  (around 390  $\mu$ L) and decrease in  $H_2S$  content (25  $\mu$ L in the gas phase), because of the differing solubility of  $H_2$  and  $H_2S$ . The estimation of sulfide content of the cell-free liquid phase (data not shown) indicated around 5  $\times$  more  $H_2S$  in the liquid phase relative to its amount in the headspace. Therefore, the extra  $H_2$  seems to be in the same range as

the drop in  $H_2S$  production. Moreover, in another experimental setup, the  $H_2S$  production of GB12131 was compared to that of the GB2131 and THOE5M strain and significantly higher  $H_2S$  formation was measured while no hydrogen could be detected.

Furthermore, in addition to  $H_2$  and  $H_2S$ , alternative electron sinks might also compete for electrons. Therefore, from these experiments it seems that  $H_2$  and  $H_2S$  productions are alternative, competing pathways for removing electrons and the amount of Hyn hydrogenase is a bottleneck to  $H_2$  evolution. This might explain the fact that elevating the amount of hydrogenase can shift the electron flow from  $H_2S$  toward  $H_2$  evolution.

Although, according to the standard redox potentials of sulfur reduction and proton reduction, the  $H_2S$  production should be favored, these values are truly concentration dependent and in vivo, the actual redox potential values may shift. Due to the complexity of the system (solid, liquid and gas phase), the actual redox potentials are difficult to predict.

In order to test the possible role of sulfide in the  $H_2$  evolution of Hyn, the THOE5M strain was cultivated in the presence of 18.4 mM elemental sulfur. On day 4, sulfite (4 mM final concentration) was added to the culture, which reacts with sulfide resulting in immediate thiosulfate formation. Conversion of sulfide into thiosulfate significantly increased the  $H_2$  production while the amount of  $H_2S$  dramatically dropped (Table 2). Therefore, thiosulfate is a much better electron source than sulfide for hydrogen evolution of Hyn.



**Fig. 2.**  $H_2S$  production of Hyn containing strains after media change in the presence of endogenous elemental sulfur as sole electron donor, under illumination. GB2131 (continuous line, □ data points) THOE5M (dashed line, ♦ data points).



**Table 2**

Hydrogen and hydrogen sulfide productions of THOE5M strain in a medium containing elemental sulfur (upper row), or after conversion of sulfide into thiosulfate by addition of sulfite (lower row). The cells were cultivated in the presence of 18.4 mM elemental sulfur without thiosulfate.

|                         | Na <sub>2</sub> SO <sub>3</sub> in the media (mM) | H <sub>2</sub> in the headspace (μl) | H <sub>2</sub> S in the headspace (μl) |
|-------------------------|---|--------------------------------------|--|
| Before sulfite addition | 0   | 19.9 ± 5.2                           | 43.6 ± 11.4                            |
| After sulfite addition  | 4   | 58.3 ± 19                            | 2.9 ± 3.4                              |

### 3.3. Both sulfur and nitrate can enhance the in vivo H<sub>2</sub> uptake of Hyn hydrogenase

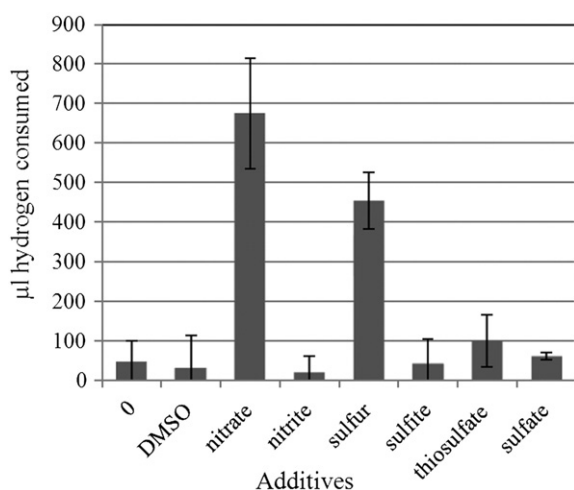
In the following experiments, we studied whether the Hyn-catalyzed hydrogen oxidation can be stimulated by various electron acceptors. The effect of several acceptors on the H<sub>2</sub> uptake of Hyn was tested (Fig. 3). Results clearly show that not only elemental sulfur but nitrate also increased the Hyn-associated H<sub>2</sub> uptake.

The draft genome sequence of the strain was determined. Genes of periplasmic nitrate reductase [KJ194603] and periplasmic sulfur reductase like molybdoprotein [KJ194601] were identified in the *T. roseopersicina* genome. The potential sulfur reductase of *T. roseopersicina* resembled (24% identity) the sulfur reductase of *A. ambivalens* [17]. In a previous study, it was shown that the activity of molybdenum-containing enzymes could be inhibited by tungstate [16].

Indeed, in *T. roseopersicina*, 2 mM sodium tungstate decreased the hydrogen-linked hydrogen sulfide production by almost 50% in the presence of endogenous elemental sulfur as exclusive sulfur source, both in illuminated and dark conditions (data not shown). This indicates the involvement of a molybdoenzyme in the hydrogen-linked sulfur reduction.

Hydrogen-linked hydrogen sulfide production could only be detected in whole cell extracts (disrupted cells containing all cellular fractions) and in pure membrane fractions of GB2131 supplemented with FAD and NADH, as was described in *A. aeolicus* [4]. Pure membrane fraction without cofactors had no detectable activity. These results confirmed the existence of an active sulfur reductase in the membrane. The role of the cofactors is still unknown.

Mutants of two candidate genes were generated, but their inactivation did not lead to the expected phenotype (data not shown). Hence, the identification of this molybdoenzyme is the next challenge for the near future.



**Fig. 3.** Hydrogen uptake of Hyn-containing strain GB2131 in darkness after media change. The cells were grown in a medium for avoiding sulfur globule formation, the test media were supplemented by various kinds of potential electron acceptors (6 mM final concentration) indicated in the figure.

The predicted nitrate reductase of *T. roseopersicina* had apparent similarity (53% identity) to the Nap-type nitrate reductase of *Rhodobacter sphaeroides*, which can also take electrons from the membrane redox pool via cytochrome bc1 and periplasmic cytochromes [20]. Since, the electron donors of nitrate and sulfur reductases are usually quinones directly or via cytochromes [17,20], the Hyn hydrogenase should somehow be involved in the hydrogen-dependent reduction of sulfur and nitrate via quinones or cytochromes.

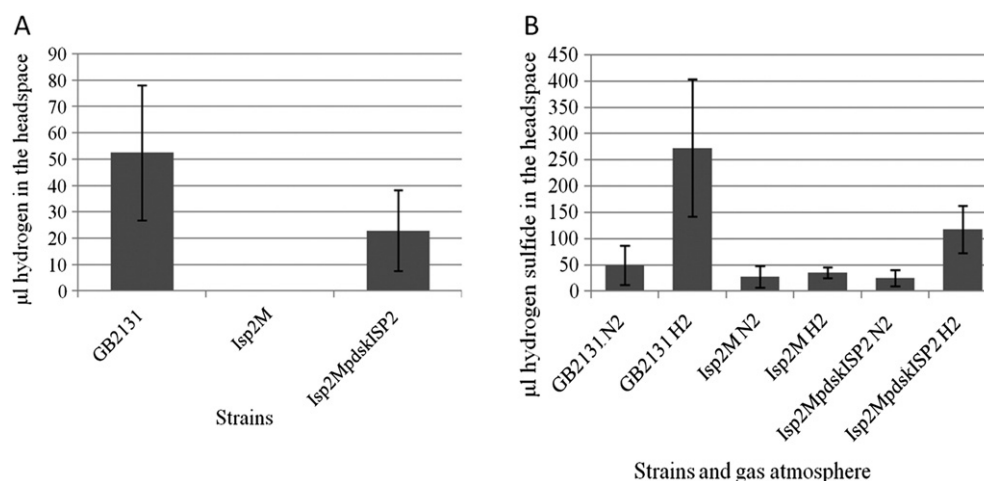
### 3.4. The Isp2 subunit is involved in the Hyn hydrogenase-linked H<sub>2</sub> driven H<sub>2</sub>S production

The Isp subunits of Hyn are essential for the in vivo H<sub>2</sub> evolution by Hyn hydrogenase. However, H<sub>2</sub> uptake was not abolished but only reduced in the absence of these subunits [2]. From these experiments, it could be concluded that the Isp1 and 2 subunits were exclusively involved in the electron donation to HynSL but in the reverse process, electrons deriving from Hyn might go through alternative routes [2]. Therefore, the role of the Isp2 in the electron transport of Hyn is still an intriguing question. It has been already observed that Hyn hydrogenase was required for H<sub>2</sub>-dependent H<sub>2</sub>S formation from sulfide [3]. We were interested in whether the Isp2 protein had any role in electron transfer from HynSL toward sulfur reduction or whether the electrons might go through a bypass route or via Isp1 as modeled in the case of *A. ambivalens* [17]. Loss-of-function Isp2 mutant (Isp2M) and Isp2-complementing (Isp2MpDSKIsp2) strains were constructed (see Materials and Methods, 2.3, 2.4 Section) and their H<sub>2</sub> evolution as well as their H<sub>2</sub>-dependent H<sub>2</sub>S formation were measured (Fig. 4).

There was no H<sub>2</sub> evolution in the *isp1<sup>+</sup>Δisp2* strain while H<sub>2</sub> evolution could be partially restored in complementation experiments: 40% complementation could be achieved (Fig. 4A). Consequently, the indispensable role of Isp2 in cellular H<sub>2</sub> evolution [2] was confirmed. In the absence of Isp2 protein, the H<sub>2</sub>-driven H<sub>2</sub>S formation could not be observed (Fig. 4B). Using the Isp2 complemented strains, again, 40% of the activity of the wild type strain could be recovered. Therefore, Isp2 protein is an essential component of the electron transfer pathway from Hyn hydrogenase to sulfur reduction. These findings do not coincide with the model established for *A. ambivalens* harboring similar hydrogenase complex [17]. In their model, Isp1 is the electron transporter between the HynSL and the sulfur reductase. The two strains are taxonomically distantly related, *T. roseopersicina* is a Gram (–), purple sulfur photosynthetic bacterium, while *A. ambivalens* is a sulfur-metabolizing Crenarchaeota strain belonging to the *Sulfolobaceae* genus. Consequently, in spite of similarities, alternative routes in metabolic, redox pathways might not be surprising.

### 3.5. The in vivo H<sub>2</sub> evolution of Hyn is light dependent

In order to get a deeper insight into the connection between the photosynthetic electron transport chain and Hyn hydrogenase, the light dependence of H<sub>2</sub> evolution of the GB2131 and THOE5M strains was examined. The cells were grown in the presence of 36 mM thiosulfate and 23 mM sodium hydrogen carbonate and the H<sub>2</sub> evolution was measured between day 3 and day 6. Upon illumination, the GB2131 and the THOE5M strain produced 50 μl and 370 μl hydrogen, respectively. In contrast, neither of these strains produced H<sub>2</sub> in the dark. Furthermore, no Hyn-linked H<sub>2</sub> evolution could be observed in the presence of sulfite (Fig. 1), pyruvate, glucose, succinate and malate in darkness (data not shown). As mentioned above, the metabolism of these substrates could not provide electrons for HynSL under illumination either. Thus, one could conclude that H<sub>2</sub> evolution of Hyn hydrogenase is tightly light dependent and can occur in the presence of the above-mentioned sulfur compounds. It should be mentioned that Hox1 could produce H<sub>2</sub> in the dark [21] and these facts together clearly indicated the distinct physiological roles of these two hydrogenases.



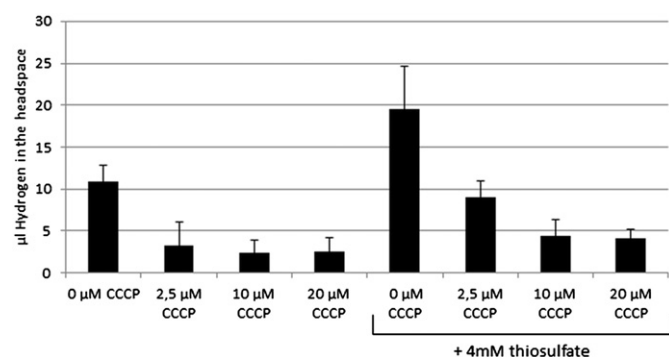
**Fig. 4.** Effect of *isp2* deletion on the HynSL-linked A)  $H_2$  evolution, B)  $H_2$ -driven  $H_2S$  formation.  $H_2S$  production of cultures was monitored in the presence of elemental sulfur under  $N_2$  or  $H_2$  atmosphere (indicated in the label of x axis). For the names of the strains see Table 1. For the details of the experiments see Materials and Methods.

Sodium thiosulfate assimilation and sulfur oxidation are tightly coupled to the photosynthetic electron transport [6,12]. Taking into account of our data and these findings, it is likely that Hyn is also linked to the photosynthetic electron transport processes.

### 3.6. Proton motive force is used for Hyn-catalyzed $H_2$ evolution

The majority of photosynthetic energy is used for proton motive force (pmf) generation via cyclic electron transport. Therefore, the role of proton motive force was tested in the  $H_2$  evolution of HynSL. In these experiments, the effect of CCCP uncoupler on  $H_2$  production in the presence of elementary sulfur and thiosulfate was examined.  $H_2$  evolution was remarkably decreased in the presence of CCCP, which strongly indicates the role of proton motive force in the energetic coupling (Fig. 5). Over 10  $\mu$ M CCCP no further inhibition could be observed and some pmf-independent but light-dependent  $H_2$  evolution could still be measured (around 20–25% of the non-inhibited sample).  $H_2S$  production was also investigated in the presence of uncoupler but no inhibition could be noticed (data not shown).

These findings suggest that Hyn hydrogenase is connected to the photosynthetic electron transport chain two ways: both pmf-dependent and independent ways.



**Fig. 5.** Role of uncoupler, CCCP, in the sulfur and thiosulfate driven  $H_2$  evolution in THOE5M strain. The  $H_2$  evolution was monitored in the presence of endogenous elemental sulfur and additives. The examined period was only one day due to the spontaneous decomposition of CCCP and the experiments were performed in smaller volume with fewer cells than in the previous experiments. Therefore, the absolute values of the  $H_2$  content cannot be compared to the data shown in other figures.

### 3.7. Photosynthetic electron transport inhibitors reduce $H_2$ evolution of Hyn

In order to elicit whether Hyn hydrogenase is coupled to the membrane electron transport chain, the effects of various electron transfer inhibitors (terbutryne, atrazine, antimycin A + myxothiazol cocktail) on the  $H_2$ -dependent  $H_2S$  and/or  $H_2$  formation were tested. In the presence of 200  $\mu$ M terbutryne, the  $H_2$ -linked  $H_2S$  production yield dropped to 1/3 as compared to the value obtained with inhibitor-free samples. Terbutryne is a competitive inhibitor of the  $Q_B$  site in the reaction center; an alternative binding site has not been published.

On the other hand, myxothiazol/antimycin A mixtures inhibiting the quinol oxidation in cytochrome bc1 complex could reduce  $H_2$  evolution of Hyn by around 40% (data not shown). This maximal inhibition was achieved at 2.5  $\mu$ M myxothiazol and 10  $\mu$ M antimycin A concentration. At higher inhibitor concentrations,  $H_2$  production remained practically the same: around 60% of the non-inhibited samples. The myxothiazol + antimycin A cocktail did not interfere with the  $H_2$ -dependent  $H_2S$  formation. Therefore, the reduced  $H_2$  production might be due to the blockage of cyclic electron transport driven photosynthetic proton motive force generation.

These data coincide well with the physiological data and indicate that the cellular function of Hyn hydrogenase is coupled with the photosynthetic electron transport chain in two ways. Based on these and previous results, an electron transport model including the Hyn hydrogenase and the membrane redox system could be outlined (Fig. 6). The electron carrier(s) between *Isp2* and  $Q_B$  site is (are) still to be identified.

## 4. Conclusions

In this work, the metabolic and electron transport linkages of the bidirectional Hyn hydrogenase were studied.  $H_2$  evolution of Hyn hydrogenase strongly depends on the sodium thiosulfate and elemental sulfur content of the media/cells. It was demonstrated that  $H_2$  production of Hyn is light-dependent and proton motive force driven. The large part of the light energy is coupled to  $H_2$  evolution via proton motive force, but even at high uncoupler or cytochrome bc1 inhibitor concentration apparent  $H_2$  production could still be detected. In the topological picture (Fig. 6), it is suggested that the hydrogenase subunits are on the periplasmic side while *Isp2* is on the cytoplasmic side of the membrane.  $H_2$  uptake experiments revealed that the electrons deriving from Hyn-catalyzed  $H_2$  oxidation can be used for reducing either  $S^0$  or nitrate. The role of sulfide was examined in two ways. Increasing the expression level of Hyn decreases the  $H_2S$



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